Improved *Carica papaya* tolerance to carmine spider mite by the expression of *Manduca sexta chitinase* transgene

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Abstract

Papaya plants producing the tobacco hornworm (*Manduca sexta*) chitinase protein were obtained following microprojectile bombardment of embryogenic calli derived from the hypocotyls of the cultivar Kapoho. Polymerase chain reaction (PCR) was carried out to confirm the presence of the transgene. RT-PCR and a quantitative chitinase assay showed increased levels of chitinase activity in every selected transgenic line. Insect bioassays in the laboratory showed that plants expressing the *Manduca sexta chitinase* gene significantly inhibited multiplication of carmine spider mites (*Tetranychus cinnabarinus* Boisd.). Experiments conducted to evaluate reaction of the transgenic plants to natural infection by carmine spider mites showed that the *Manduca sexta chitinase* gene provided increased tolerance under field conditions.

Abbreviations: 2,4-D – 2,4-Dichlorophenoxyacetic acid; *BA* – benzyladenine; *IBA* – indolebutyric acid; *MSCH* – *Manduca sexta* chitinase; *NAA* – naphthaleneacetic acid; *NPTII* – neomycin phospho-transferase

Introduction

Papaya (*Carica papaya* L.) is an important fruit crop grown in the tropics and sub-tropics worldwide. It has a number of natural pathogens (bacteria, viruses, and fungi) and pests (aphids, leafhoppers, mites, and nematodes) (Nishijima, 1994, 2002). The most serious insect damage to papaya grown in Hawaii is caused by leafhoppers, *Empoasca* species (Ebesu, 1985) and mites including the broad mite, *Hemitarsonemus latus*, the carmine spider mite, *Tetranychus cinnabarinus* Boisd. (LaPlante & Sherman, 1976; Hill, 1983), and a relatively new invader, the papaya leaf edgeroller mite, *Calacarus brionese* (Follett, 2003).

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In Hawaii, plant mites multiply prolifically throughout the year and can cause widespread damage in a very short time. Because of their minute size, it is difficult to detect the presence of mites on plants before populations become abundant and plant damage is obvious. The various species of mites colonize different parts of the papaya plant where their feeding causes premature defoliation, reduced tree vigor that results in reduced fruit yield, and external fruit blemishes that reduce fruit market value (Nishina et al., 2000).

The carmine spider mite has the largest host range of all *Tetranychidae* species in Hawaii and is undoubtedly of greatest economic importance (Goff, 1986; Biswas et al., 2004). This mite occurs on nearly 100 species of weeds and cultivated crops including vegetables, fruit, and ornamentals (see Goff, 1986 for an extensive list of hosts).

The carmine spider mite normally completes a life cycle from egg to adult in about 1 week. All stages of this mite are present throughout the year. Reproduction is most favorable when the weather is hot and dry. Adults and nymphs feed primarily on the undersides of the leaves. Damage due to carmine spider mite feeding appears first as yellow stippling, which is the appearance of wounded tissue surrounding the feeding punctures, followed by whole canopy yellowing and senescence. The leaves eventually become bleached and discolored and may fall off. The mites tend to feed in leaf depressions often near the midrib and veins. Silk webbing produced by these mites is usually visible.

Genetic engineering has produced a number of strategies to improve plant resistance to pests and pathogens. Transgenic papaya with resistance to papaya ringspot virus (PSRV) disease has been developed (Fitch et al., 1990) and commercialized. Seeds of the transgenic papaya cultivar "Rainbow" have been distributed to Hawaii's papaya growers so the immediate threat of PSRV has been resolved. However, the cultivar Rainbow, which was derived by crossing transgenic SunUp with non-transgenic Kapoho, has several detrimental horticultural characters inherited from its parents. For example, its female parent, SunUp, is very susceptible to the leafhopper while the male parent, Kapoho, is susceptible to mites.

One strategy for using transgenes to control a wide range of pests is to target chitin metabolism. Chitin ranks among the most abundant biopolymers. It is an insoluble, structural polysaccharide that is a component in the cell walls of fungi, nematodes, and other organisms including mites (Kramer & Muthukrishnan, 1997). Because of the pivotal role played in insect growth and development by chitin and the chitinolytic enzymes, they are receiving attention for their potential development as biopesticides and microbial biological control agents or as chemical defense proteins in transgenic plants.

Insect *chitinase* genes have been introduced into plants to enhance plant resistance to insect pests. Ding et al. (1998) incorporated a chitinase enzyme from *Manduca sexta* into tobacco and showed reduced feeding damage and stunted larval growth of the tobacco budworm (*Heliothus virescens*). Constitutive expression of *Manduca sexta* chitinase in plants could have a role in improving plant resistance against other pests such as plant parasitic mites. Recently, resistance in *Bassica napus* to the diamondback moth (*Plutella maculipenis*) was enhanced by transgenic expression of the *Manduca sexta chitinase* gene combined with a scorpion toxin gene (Wang et al., 2005). On the other hand, when a different gut-specific insect *chitinase* gene from the beetle (*Phaedon cochleariae*) was constitutively expressed in potato plants, it did not directly affect nymphal mortality of *Myzus persicae*, but it did improve several biological parameters restricting this aphid's population growth (Saguez et al., 2005).

In our attempt to enhance papaya resistance to the carmine spider mite, we report transformation of a commercial variety of papaya with the gene encoding an insect chitinase (*MSCH)* from *Manduca sexta*. This is, to the best of our knowledge, the first report of expression of the insect chitinase *MSCH* gene in *Carica papaya* and the resulting dynamics of carmine spider mite populations under laboratory and field conditions.

Materials and methods

Plasmid construction

The plasmid pBI121/MSCH contains the neomycin phosphotransferase (*NPT* II) gene as a selectable marker under the control of the nopaline synthase promoter, Nos 5′, and the Nos 3′ terminator. The *Manduca sexta chitinase* gene *MSCH*, kindly provided by Dr. S. Muthukrishnan from Kansas State University, is under the control of the constitutive *CaMV 35S* promoter and a second Nos 3′ terminator.

Plant transformation

The biolistic gene gun was used to introduce the transgene vector into the papaya cultivar Kapoho. The methods used were adapted from Fitch et al. (1990). Embryogenic calli were used as a target. Bombarded embryogenic calli were selected on media containing 100 mg ml^{-1} Geneticin (G418) (Zhu et al., 2004b). The medium used for maintenance of tissue culture material was 4.3 g l^{-1} MS salts, 30 g l^{-1} sucrose, 0.1 g l^{-1} myo-inositol, 1 ml l^{-1} MS vitamin IV, pH 5.7. For shoot production, 0.1 mg l^{-1} naphthaleneacetic acid (NAA) and 0.1 mg l^{-1} benzyladenine (BA) were

added. To encourage rooting, 0.2 mg l^{-1} indole-3-butyric acid (IBA) was added. Selected primary transformants were transferred to rooting media and then to potting soil (Sunshine #4 mix, United Agricultural Supply Company).

Transgenic plant multiplication and growth conditions

Putatively transformed plants were multiplied by using the micropropagation methods of Fitch (1993). Specifically, after 3 months selection on antibiotics, the putatively transformed callus produced plantlets on half-strength MS medium with 3% sucrose and containing 0.2 mg l^{-1} BA and 0.2 mg l^{-1} NAA. Proliferating shoots were cut into nodal sections and subcultured on the same medium once a month. Roots were initiated by placing freshly cut shoots on MS medium containing 2 mg l^{-1} IBA for 7 days, followed by removal to MS medium containing no growth regulators. Rooted plants were potted in sterilized vermiculite wetted with half-strength MS medium until dense masses of roots developed. Plants were transplanted into 4 l pots containing steam-sterilized potting soil for growing in the greenhouse at 25°C and relative humidity ranging from 30 to 60% under natural daylight for about 3 months, at which time plants were subjected to molecular and insect bioassay evaluations.

Polymerase chain reaction (PCR) and reverse transcriptase (RT)-PCR analysis

Fully expanded leaves of approximately 3-month-old, greenhouse-grown papaya plants were collected, immediately frozen in liquid nitrogen, and stored in a −80°C freezer until extracted. Genomic plant DNA was isolated using the method of Dellaporta (1994). The primers used to amplify the *NPT* II gene were 5′-AGAGGCTATTCGGCTA TGAC-3′ and 5′-GTCAAGAAGGCGATAGA AGG-3′. A 800 bp *MSCH* fragment was amplified using a pair of primers 5′-GAGCATTCATCA GGGTTGGT-3′ and 5′-AGAAGCTGGCTTAG CTGTGG-3′. Primers for actin, 5′-ACTACGA GTTGCCTGATGGA-3′ and 5′-AACCACCA CTGAGCACAATG-3′, were also used as a positive control. The actin primers, based on papaya actin partial sequence (accession AY906 938), amplified a 200 bp fragment. Both *MSCH*

specific and actin primers were used at 1 μM concentration. A total volume of 50 μl was prepared for use in a Bio-Rad iCycler® thermocycler. The conditions were 95°C for 2 min followed by 30 cycles at 95°C for 30 s, 60°C for 30 s, 72°C for 45 s with a final extension at 72°C for 10 min.

Total RNA was extracted from the same leaf samples as for PCR according to the methods of Verwoerd (1989). RT-PCR was carried out using the same *MSCH* specific and actin primers, ImProm-II^{TM} reverse transcriptase (Promega). DNAse-treated total RNA (364 ng) was used for the reverse transcription reaction and 18 ng was used for subsequent PCR amplification. The same amplification protocol was used for PCR as stated above. As a control, RNA samples without reverse transcriptase (RT minus) were included to be sure that RNA samples were not contaminated with genomic DNA.

PCR or RT-PCR product was fractionated by electrophoresis in 0.8% agarose gel. For Southern blot hybridization, the agarose gel was alkali-blotted onto Hybond N+ membranes (Amersham) according to the manufacturer's instructions. Blots were probed with an 800 bp *MSCH* coding sequence that was generated by PCR using the same *MSCH* gene specific primers. The chemifluorescent probe was labeled with AlkPhos Direct® (Amersham) according to the manufacturer's instructions. Southern blot hybridization was carried out as described by Southern (1975) and Sambrook and Russell (2001).

Quantitative chitinase activity assay

Chitinase activity was determined using a colorimetric assay adapted from the method of Ueno et al. (2004). Total soluble protein was extracted from a pool of the four youngest fully expanded leaves from 3-month-old potted plants by grinding coarsely chopped leaf blades (avoiding the midrib) in liquid nitrogen with a mortar and pestle. The ground powder was homogenized in 100 mM potassium phosphate buffer (pH 7.0) with the addition of a protease inhibitor cocktail (complete mini EDTA-free, Roche). The slurry was centrifuged at 10,000 × g for 10 min and the supernatant decanted. Protein concentration was quantified using the method described by Bradford (1976). For the chitinase assay, the supernatant was diluted to 0.5 μg protein in a volume of 200 μl

H_2O to which was added 100 μl of 2 mg/ml carboxymethyl Remazol Brilliant Violet chitin (CM-Chitin-RBV, LOEWE Biochemica GmbH, Germany) and 100 μl of 0.2 M sodium phosphate buffer (pH 6.8). The reaction mixture was incubated for 2 h at 37 °C then stopped by addition of 100 μl of 2 M HCl and incubated on ice for an additional 15 min. The sample was centrifuged at 12,000 $\times$ g for 5 min to pellet the remaining non-digested substrate and the supernatant was decanted. Absorbance of the supernatant was measured at 540 nm using a MRX microplate reader (Dynatech Laboratories). A blank tube containing all reactants except the plant enzyme extract was used as a background control. The background value was subtracted from all sample readings to give a change in absorbance at 540 nm value (Δ540 nm). Enzyme activity was expressed as Δ540 nm μg protein^{-1} min^{-1}. For each transgenic line, three separate protein extractions were prepared from pooled leaf material and three replicate samples were taken from each protein extract for enzyme assays.

Insect bioassay

A laboratory-based bioassay was conducted using the *MSCH* transformed lines. Potted plants of approximately 6 months of age and 1 m in height were used. The plant material was maintained under warm white fluorescent light, PAR (photosynthetically active radiation) of 14 μE m^{-2} s^{-1}, with temperature 20 $\pm$ 7°C and relative humidity 56 $\pm$ 19%. The experiment was designed as a complete randomized block with four replicates of each plant sample. The distance between plants was 0.3 m. Five MSCH transgenic lines plus the non-transformed control cultivars Kapoho and Sunset were used. Each plant was inoculated with 20 adult mites and monitored over the next 10 weeks to score the number of adult mites on each leaf and the number of leaves abscised from the plants. Each leaf was divided into six sections for counting individual adult mites by naked eyes; the numbers from each section were combined. Juvenile mites were not counted as they are not easily visible without lens magnification.

Field trials were conducted on five replicates per line at the Kunia field station on the leeward plain of the island of Oahu, Hawaii, where mite infestation is characteristically heavy. Data were collected by counting the carmine spider mite population on two young, fully expanded, leaves and two older leaves from plants about 12-months-old and spaced about 1.5 m apart. Other non-target insects including fruit flies, spiders, ladybugs and scales, were also counted on these leaves to see if their numbers were affected.

Data analysis

The mite count and chitinase activity data were analyzed using the general linear model (GLM) procedure of the SAS (Statistical Analysis System Inc., Cary, NC). When treatment effects were significant ($p < 0.05$), means were separated using the least significant difference (LSD) ($p = 0.05$).

Results

Selection of transgenic lines

Twenty plates (each plate contains approximately 1 g fresh weight) of embryogenic callus were used for transformation. Nineteen independent lines were selected by survival on G418 (100 mg l^{-1}) selection medium and confirmed as transgenic by PCR using primers specific for *NPT* II (Figure 1a). PCR amplification of the *NPTII* gene in all selected lines indicated that the selection was carried out with high stringency since there were no escapes. For this experiment, the transformation rate using G418 was approximately one transformed line per bombarded plate, which is consistent with our earlier published reports using the bombardment method (Zhu et al., 2004a, b).

The presence and expression of the *MSCH* gene were confirmed by RT-PCR amplification using total RNA extracted from putative transgenic plants. Fragments of the expected sizes (800 bp fragment using *MSCH* specific primers) were present in the five transgenic lines shown (Figure 1b) but absent in the Kapoho control. As a PCR reaction control, actin primers were used to amplify the expected 200 bp product from all lines tested, including the non-transformed Kapoho control (Figure 1c).

The presence and expression of *MSCH* gene in two representative transgenic lines, T-24 and T-23, were further confirmed by hybridizing RT-PCR
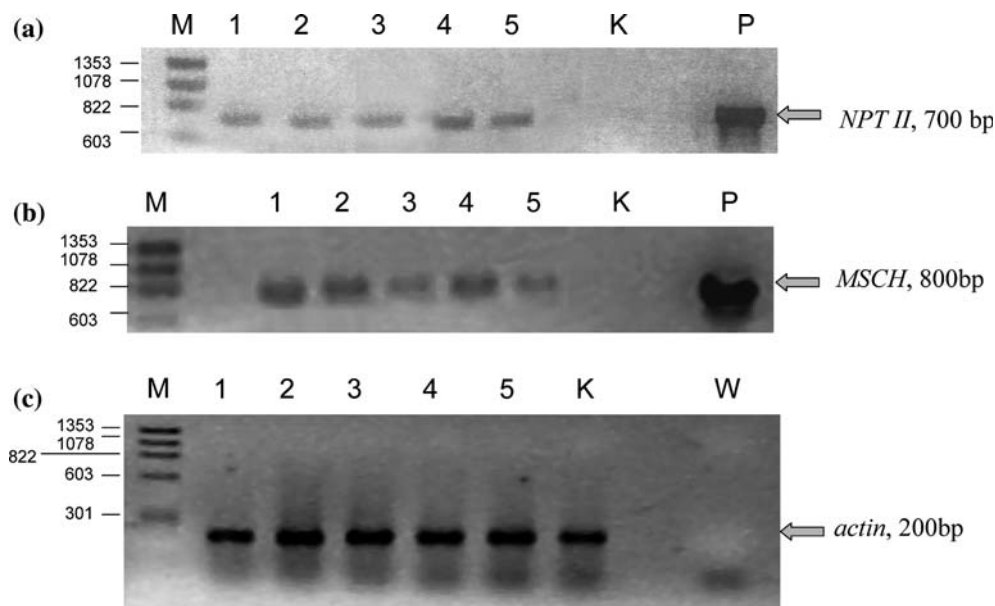
Figure 1. Molecular analysis of MSCH transformed lines. (a) Agarose gel electrophoresis of the PCR products using the *NPTII* gene specific primers. (b) Agarose gel electrophoresis of RT-PCR products using the *MSCH* gene specific primers. (c) Agarose gel electrophoresis of RT-PCR products using the actin primers. Lanes: M, molecular weight marker; 1–5 transgenic papaya lines (T-9, T-14, T-18, T-23, and T-24); K, wild-type Kapoho control; P, plasmid; W, water control. The numbers on the left indicate the positions of the molecular weight marker in bp.

product with *MSCH* specific probe labeled with chemifluorescent AlkPhos Direct® (Amersham) (Figure 2). The *MSCH* specific probe hybridizing with the RT-PCR fragment in T-24 and T-23, but not with non-transformed control, indicated that
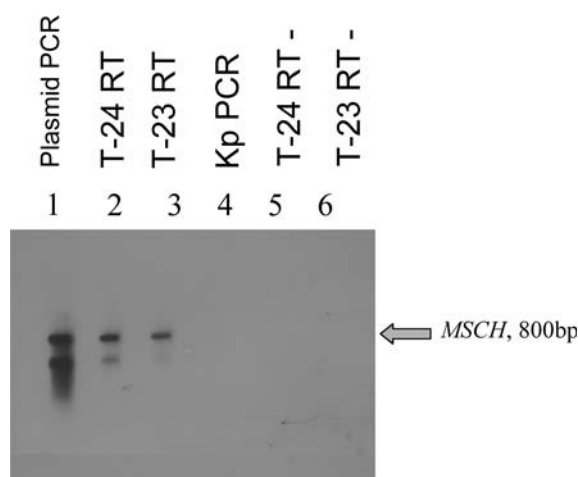

Figure 2. Hybridization of RT-PCR and PCR products blotted and probed with the *MSCH* gene. Lanes: 1, Plasmid control; 2 and 3, transgenic plants T-24 and T-23 with reverse transcriptase; 4, non-transformed Kapoho control; 5 and 6, transgenic plants T-24 and T-23 without reverse transcriptase.

the RT-PCR product is *MSCH* gene specific. When DNase-treated RNA samples without the addition of reverse transcriptase enzyme, were used as a substrate (lanes T-24 RT- and T-23 RT-), the absence of product confirmed that the RNA samples were not contaminated with genomic DNA.

Chitinase activity was determined on leaf extracts from MSCH lines T-2, T-9, T-14, T-18, T-23, and T-24. The chitinase activities of all six transgenic lines are significantly greater than that of the Kapoho parent (Figure 3). The increased chitinase activity ranged from 9 (T-18) to 52% (T-24) of Kapoho control.

Laboratory insect bioassay

Over the 10-week period averaged for the non-transformed cultivars (Kapoho and Sunset) and five *MSCH* transformed lines (T-23, -14, -9, -18, and -24), the leaf number remained constant and the mite population increased from an initial inoculation of 560 adults to a maximum of 27,000 on all plants at week 7 (Figure 4). The mite population dropped to 9000 in week 8, to 2000–3000 in week 9, and remained at this level
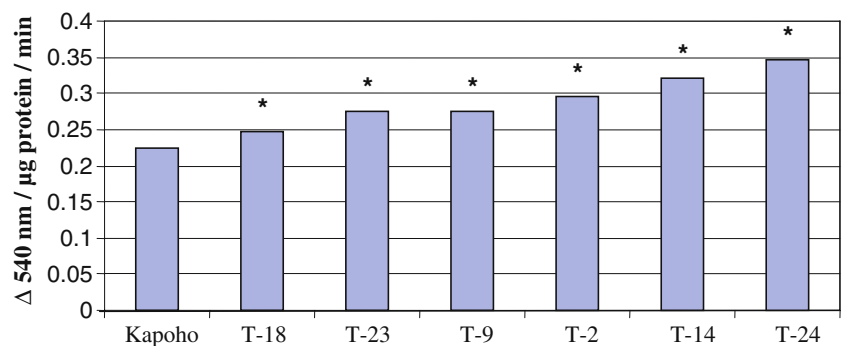
Figure 3. Chitinase activities in protein extracts of six transgenic papaya lines and their donor cultivar, Kapoho. Values presented are the averages for three separate protein extractions from pooled leaf laminae. *Means significantly different from Kapoho, non-transformed control ($p \leq 0.05$).
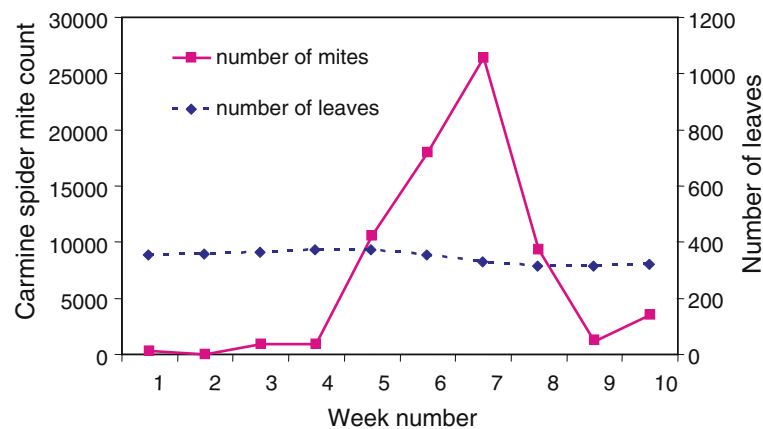

Figure 4. Measured mite population and total leaf number on plants over a 10-week period. Four replicate plants of five MSCH lines and the Kapoho and Sunset control varieties were maintained under laboratory conditions. Each plant was inoculated with 20 adult mites. Over a 10 week period the total number of leaves and adult mites were counted for all plants.

thereafter. At the peak of the mite population in week 7, the number of mites on the transgenic lines T-23, -14, -9, -18, and -24 was compared with the number on the non-transformed controls Kapoho and Sunset (Figure 5a). Sunset, the more susceptible control, had the highest mite population, averaging 180 mites per leaf, while Kapoho, the less susceptible parent, had fewer mites, averaging about 100 mites per leaf. Five transgenic lines showed a wide range of mite numbers with T-24 showing significantly fewer mites compared to its non-transformed control, Kapoho, while T-23 and T-14 had significantly higher number of mites than Kapoho but significantly fewer than Sunset. The mite populations in T-9 and T-18 plants were similar to that on Kapoho.

The damage to leaves only weakly correlated with the number of mites on the transformed and non-transformed lines. Both of the non-transformed cultivars Kapoho and Sunset (data not shown) lost more leaves than did the transformed lines (Figure 5b). Kapoho lost almost eight leaves per plant while T-9, T-14, T-23, and T-24 lines all gained one or two leaves per plants, even though T-23 and T-14 had significantly higher mite populations. It appeared that transgenic plants were much more vigorous with less damaged leaves than the non-transformed lines, even when the transformed plants had more mites (Figure 6). Overall, it appeared that mites preferred feeding on the non-transformed cultivars. This observation is backed by a previous
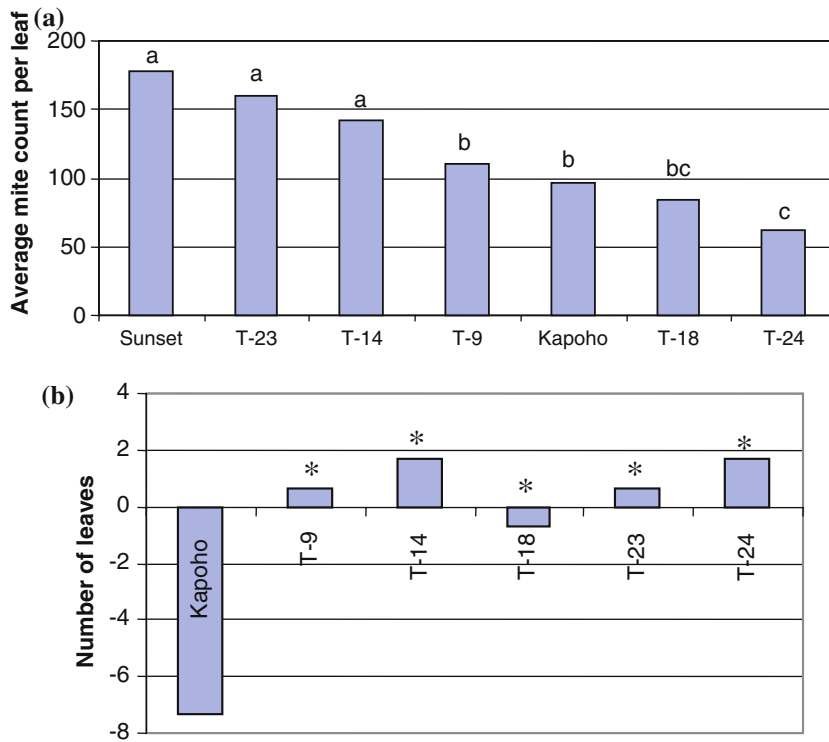
Figure 5. Laboratory insect assay. (a) Mite population per leaf at 7-week post inoculation. In the laboratory bioassay a count of adult mites and the number of leaves on each plant was made. The total number of adult mites counted for all plants in each treatment was divided by the total number of leaves on the plants. Values labeled with different letters (a,b,c) are significantly different ($p < 0.05$) determined by ANOVA and the Waller-Duncan K-ratio t-test. Sunset is a susceptible variety, Kapoho is a moderately tolerant variety. T-23, T-14, T-9, T-18, and T-24 are Kapoho lines transformed with the *MSCH* gene. (b) Change in number of leaves at 10-week post inoculation. Kapoho is the non-transformed control. T-9, T-14, T-18, T-23, and T-24 are Kapoho transformed with the *MSCH* gene. *Means significantly different from the non-transformed control.

Kapoho   T-14

Figure 6. Representative papaya leaves inoculated with carmine spider mites. The photo was taken 7 weeks after initial mite inoculation.

"preference test" where a mixed population of non-transformed Kapoho or Sunset plants had a significantly higher number of adult mites than did the *MSCH* transformed lines when all were naturally infected in the greenhouse. The transformed line T-24 consistently had fewer mites than did the Kapoho control (Figure 5a) and averaged two new leaves per plant. Comparing the two non-transformed cultivars showed that the number of mites on Sunset was consistently higher than on Kapoho. This finding corroborates field observations (R. Ming, personal communication).

The result of mite counts at 7 weeks post inoculation was similarly observed at the 8-, 9-, and 10-week post inoculation, except the total mite count was lower. Nevertheless, the pattern of mite distribution across different plant lines was the same (data not shown). At week 8, control plants started to senesce due to the loss of leaves.

Field trial

A count of all mites and eggs found on the young plant leaves revealed no significant difference between the number found on the *MSCH* transformed lines and the non-transformed cultivars. In the field trial (Figure 7), as was observed in the laboratory trial, adult mites preferred the older leaves. Also, consistent with laboratory inoculation experiment, the non-transformed Kapoho controls had fewer mites than the less-tolerant Sunset (Figures 5a, 7). Interestingly, in the field where natural infestation occurred, the number of mites counted on all five transformed lines (T-9, -14, -18, -23, and -24) was significantly lower than for Kapoho. This suggests that mites prefer to feed on the control plants or that the mites on the *MSCH* plants were not as fecund. The leaves of the transgenic lines showed less mite damage than the controls, as similarly observed in Figure 6. The count of other non-target insects indicated they were not affected by the MSCH transgene, but the numbers were too small for statistical analysis.

Discussion

In the present work, a chitinase activity assay was used to determine whether the recombinant protein was biologically active. The chitinase assay showed enzyme activity in the non-transformed papaya parent as well as higher activity in six plant lines that had been shown by RT-PCR to be transformed with the *MSCH* gene construct (Figure 3). Chitinase activity in the non-transformed control was not surprising since chitinases are known to be widely distributed among bacteria, fungi, plants, and animals where different forms of chitinase can be involved in different functions (Cohen-Kupiec & Chet, 1998). In plants and animals, chitinases mainly play a role in defense of the organism against pathogen attack, but they can also be involved in various growth and developmental processes. Since the papaya endogenous chitinase has been characterized (Azarkan et al., 1997) and no plant chitinase is known to have insecticidal activity (Kasprezewska, 2003), we can attribute the insecticidal activity of the transformed lines to the expression of the *MSCH* gene. All tested MSCH lines were found to have higher chitinase activity and greater mite tolerance when compared to that of the parent Kapoho (Figures 3, 5, 7). In the laboratory and field assays it would appear that transformed lines showing similar chitinase activity produced similar control of mites. For example, when plant growth was examined in the
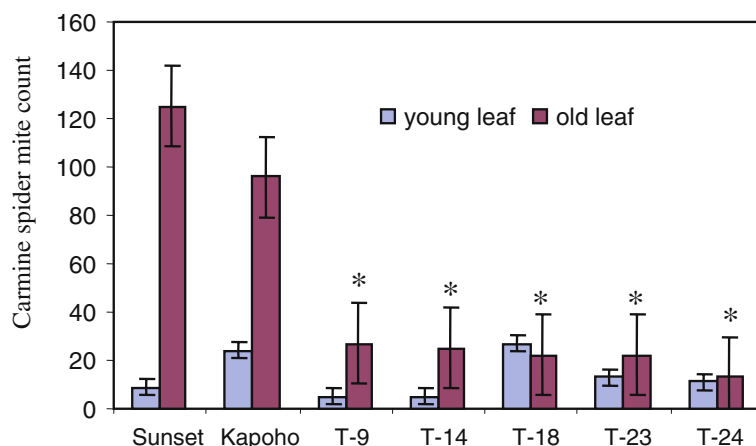

Figure 7. Field data of mite population 9 months after planting. Field was naturally infested with carmine spider mites. Adult mites were counted on two young and two old leaves for each plant at 9 months after planting. Five replicate plants were tested for each line. Bars represent standard error. *Means significantly different from Kapoho, non-transformed control.

laboratory assay it was found that T-9 line produced a few new leaves, lines T-14 and T-24 both produced more. In part this might be explained by the fact that T-9 had 1.2 times Kapoho chitinase activity while T-14 and T-24 had 1.4 and 1.5 times as much activity. Of the six lines tested in the chitinase activity assay, T-24 showed the highest activity and was the most resistant based on the average mite count/leaf.

In our laboratory evaluation, each plant was inoculated with 20 adult mites and the population measured each week thereafter. The total mite population on all plants peaked at week 7 from initial population of 560 to 27,000. The number of mites then decreased and remained constant at 2000–3000 after week 8. This stable count of mites coincided with the death of control plants, where Kapoho and Sunset lost most of their leaves by week 8 and the plants started to die thereafter. The significant decrease in total mite population appeared to be due to the loss of the more favorable food sources, the non-transformed Kapoho and Sunset.

Naturally, spider mites colonize individual leaves and form colonies that may move or migrate in the canopies of plants (Croft & Jung, 2001). Movement of mites in a colony or patch occurs frequently, is mostly by ambulatory means, and has a low risk for mortality (Strong et al., 1999). Larger movement of mites from one isolated plant to another (interpatch) occurs less often and has more risk for mortality than intrapatch movement (Nachman, 1988). Interpatch movement is affected by many factors including temperature and humidity, wind and spatial structure of the patch, and loss of food supply (Strong et al., 1997, 1999).

Mite movement may account for differences in populations on the transformed and non-transformed lines in the laboratory. In the laboratory mite feeding test, the MSCH lines appeared more tolerant than the non-transformed control Kapoho plants since they withstood mites and produced a greater number of new leaves than did the controls. Line T-24 appeared to be the most tolerant as it consistently had a lower mite population than did Kapoho. On the contrary, T-23 and T-14 had significantly higher mite counts. This may be because the control plants lost more leaves and remaining leaves were not as healthy as ones on the transgenic plants, with

the net result that mites moved from the dying non-transformed control plants onto the transgenic plants. Even though lines T-23 and T-14 had significantly higher mite counts than the controls, the plants added additional leaves over the 10-week period while control plants all died by the end of 10 weeks. This suggests that transformed plants withstood large mite infestations and were more tolerant to them. This in turn suggests that the higher level of tolerance could be due to reduced feeding activity of the mites. Since only adult mites were counted and the juvenile mites were too small to count with naked eyes in these experiments, it would be interesting to know the reproduction rates of mites that fed on control plants vs. the transgenic plants. Perhaps results would have been different if a larger population of plants was used since control plants lost more leaves and their remaining leaves were senescing compared to those of transgenic plants. It appears that mites migrated onto the transgenic plants after the controls were no longer a food source. In addition, isolation or increasing spacing among the plants to restrict mite migration might be better for understanding the response of mites to the MSCH transgene.

In the field trial, the plants were older and larger (12-month-old compared to 6-month-old in the laboratory trial) and the spacing between them was much greater (1.5 m compared to 0.3 m). Our result from the field trial showed that every MSCH transformed line had fewer mites than did the controls. The distinct difference in mite population in the laboratory trial was probably due to the ability of mites to migrate across the reduced spacing, lack of predators, and the rapid depletion of non-transformed control leaves. Once mites infested the field plants, they would be less likely to migrate since the migration distance was much greater and there was considerably more leaf area on the older plants.

The actual mode of action of the *Manduca sexta* chitinase on insects is unknown (Ding et al., 1998). The peritrophic membrane that encloses food in the mid and hindgut has been suggested as a target. Normally, insects would be exposed to the chitinase enzyme only at specific periods of the molting process. Exposure to chitinases at the wrong time might be detrimental to insect development (Kramer & Muthukrishnan, 1997). Whatever the mechanism, it would appear that papaya

plants expressing the *Manduca sexta* chitinase have improved resistance to mites because mites prefer not to feed on *MSCH* lines when there are non-*MSCH* plants available. It would be interesting to know if this aversion to feeding on *MSCH* plants would hold in larger plantings of only *MSCH* transformed lines and how this trait is sexually transmitted to subsequent generations. *Manduca sexta* chitinase has potential to be used as an alternative or addition to *Bt* or other insecticidal genes in the control of insects that cause significant yield losses in papaya.

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